



Impacts of a highway on the population genetic structure of a threatened freshwater turtle (*Glyptemys insculpta*)

^{1,2,*}Alexander J. Robillard, ²Sean Robinson, ²Elizabeth Bastiaans, and ²Donna Vogler

¹Chesapeake Biological Laboratory, University of Maryland Center for Environmental Science, Solomons, Maryland, USA ²Department of Biology, State University of New York College at Oneonta, Oneonta, New York, USA

Abstract.—Genetic partitions for members of the family Emydidae often correspond with both natural and anthropogenic landforms. For semi-terrestrial turtles, clear negative impacts are associated with habitat fragmentation via roadways, such as loss of breeding individuals, increased inbreeding, and decreased migration. The Wood Turtle (*Glyptemys insculpta*) is a Species of Special Concern in New York and native to the central portion of the state, where Interstate Highway 88 was constructed in the 1970s. To examine possible impacts of the highway on local populations, a museum collection of Wood Turtles that predates road construction was used. Specifically, microsatellite markers were used to compare historic ($n = 38$) and contemporary ($n = 26$) Wood Turtle DNA from opposite sides of the highway. The measured parameters were inbreeding (F_{is}), differentiation (F_{st}), number of breeding individuals (N_b), migration (m), and overall population genetic structure. The populations on either side of the highway were predicted to have become more differentiated and inbred over time, and migration was predicted to decrease over time. Overall, populations on either side of the interstate were historically a single population, had a greater number of breeding individuals, and were less differentiated. No change in inbreeding was found across time. These findings suggest there is more migration, running north to south between the two populations, likely attributable to the directionality of the flow associated with local creeks. Further research examining these two separate populations within the context of the entire state is necessary to determine whether they should be treated as separate Conservation Units.

Keywords. Emydidae, habitat fragmentation, roadways, microsatellite, population structure

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Introduction

On a global scale, amphibians and reptiles are in decline due to pressures which include climate change, unsustainable harvest, habitat loss, and habitat degradation (Gibbons et al. 2000). Among all reptile and amphibian species, members of the order Testudines are particularly vulnerable to increased decline when faced with increasing anthropogenic disturbances, such as road mortality and illegal harvesting (Lieberman 1994; Garber and Burger 1995; Wood and Herlands 1997; Williams 1999; Levell 2000; Gibbons et al. 2000; Gibbs and Shriver 2002; Steen and Gibbs 2004; Gibbs and Steen 2005; Steen et al. 2006; USFWS 2015). Nearly half of all turtle species are currently categorized as threatened or endangered (Rhodin et al. 2011). Terrestrial turtles are generally perceived as poor long-distance dispersers. Limitations to dispersal enable habitat fragmentation, which can put populations at risk of extinction due to demographic and genetic diversity loss (Gibbons et

al. 2000). In North America, several case studies have suggested that anthropogenic disturbances, particularly roadways, have direct negative impacts on freshwater turtles by skewing sex ratios and increasing the mortality of migrating individuals (Buhlman and Gibbons 1997; Wood and Herlands 1997; Williams 1999; Levell 2000; Gibbons et al. 2000; Gibbs and Shriver 2002; Steen and Gibbs 2004; Gibbs and Steen 2005; Steen et al. 2006; USFWS 2015).

When assessing the negative impacts of fragmented populations, genetic markers can identify dispersal pathways and population diversity (Lamb et al. 1989; Galbraith et al. 1995). For example, studies focusing on turtle populations have found river drainages and intermontane basins to be barriers to gene flow (Gibbs and Amato 2000). Similarly, several studies have identified relatively high allelic diversity in Wood Turtle populations when compared to other species (Gibbs 1993; Tessier et al. 2005; Amato et al. 2008; Castellano et al. 2009; Spradling et al. 2010). Given

Correspondence. *ajrobill@umd.edu

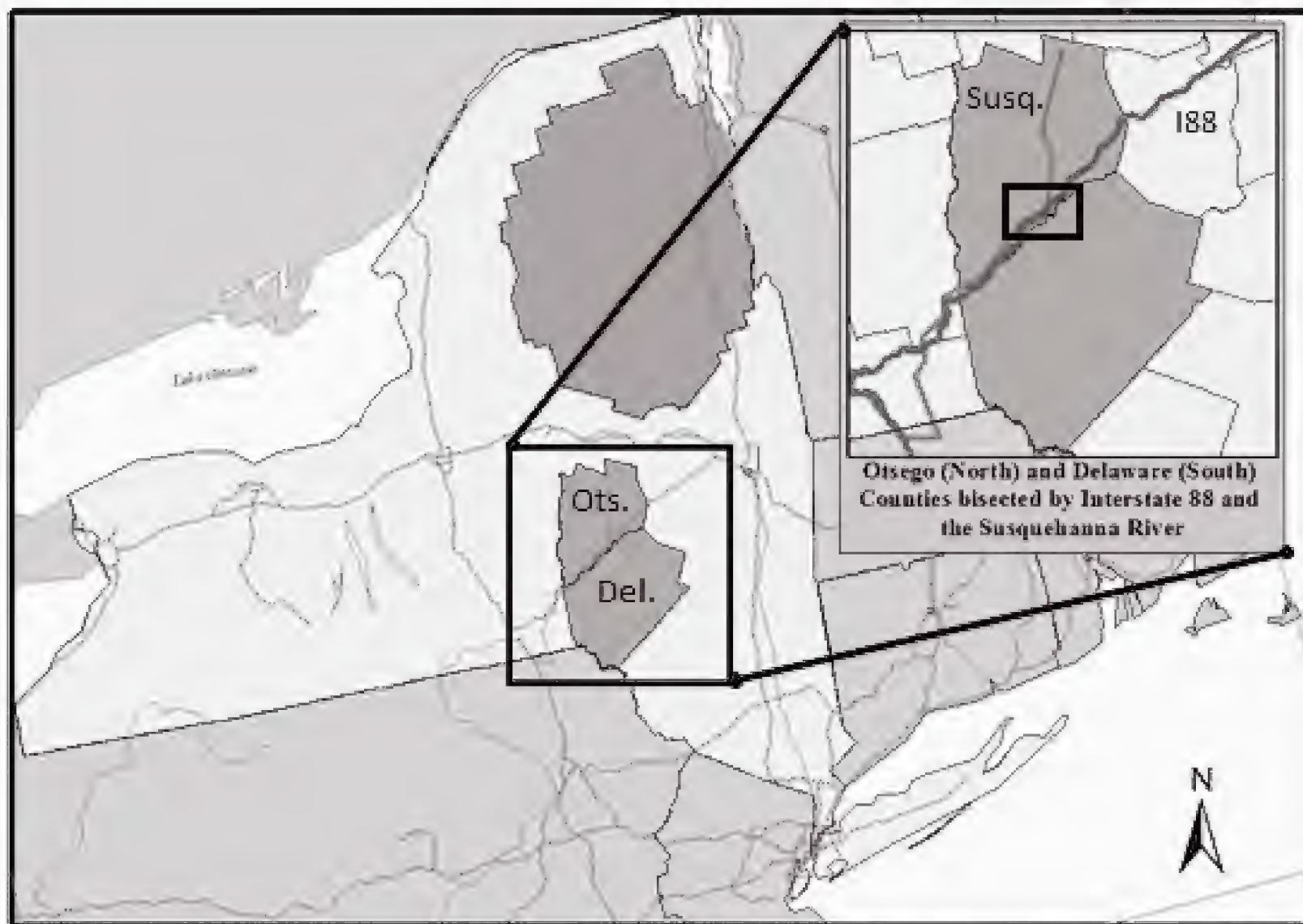


Fig. 1. Study area. Interstate Highway 88 (I-88) and the Susquehanna River (Susq.) bisect Otsego and Delaware Counties, New York, USA.

their poor long-distance dispersal ability, populations of freshwater turtles could be at increased risk from habitat fragmentation via natural or anthropogenic barriers, which may result in loss of genetic and demographic connectivity (Gibbons et al. 2000; Gibbs and Amato 2000). Specifically, turtles which do attempt to move long distances within a fragmented landscape, as females often do for nesting, may be at greater risk of dispersal related mortality, e.g., roadkill (Gibbs and Shriver 2002; Steen and Gibbs 2004). Road mortality driven by habitat fragmentation is believed to be the culprit for the gradual skewing of sex ratios and the general decline of female turtles among many freshwater turtle species throughout the United States (Steen and Gibbs 2004; Gibbs and Steen 2005; Steen et al. 2006).

Known to disperse both long and short distances (Harding and Bloomer 1979) throughout its range, the Wood Turtle (*Glyptemys insculpta*) is a Species of Special Concern in New York State, which may be at risk from the demographic and genetic impediments associated with habitat fragmentation (Gibbons et al. 2000; Breisch and Behler 2002; Tuttle and Carroll 2003; Arvisais et al. 2004; Sweeten 2008). Limitations to dispersal within fragmented landscapes are believed to contribute to the decline of wetland-dependent turtles (Gibbs 1993; Tessier et al. 2005; Amato et al. 2008; Castellano et al. 2009; Spradling et al. 2010). Although previous studies have examined Wood Turtle population genetics, none have focused on the potential impacts that anthropogenic

habitat fragmentation could have on regional populations (Tessier et al. 2005; Amato et al. 2008; Castellano et al. 2009; Spradling et al. 2010).

Between 1974 and 1980, Interstate Highway 88 (I-88, 193 km) was built across eight Central New York counties (Fig. 1), including Otsego and Delaware counties (Associated Press 1986; Edwardsen 1989). Prior to this, from 1958–1968, Dr. John New collected and dry-preserved Wood Turtles ($n = 300$) from across New York state, including sites north and south of I-88. Using this historic data set in conjunction with contemporary data, this study examines the potential impacts of building a large interstate highway on a vulnerable turtle species over a 60-year period. Over that same 60-year period, New York State has become more populated, according to U.S. Bureau of Census data for 1960–2010. Given that wetland habitat size and connectivity degrade with increased human activity (Gibbs 2000), and that such disruption of wetland mosaics can have dramatic negative impacts on semi-terrestrial turtle populations (Gibbs and Shriver 2002), declines are expected to be observable on the genetic scale. Specifically, an increase in genetic differentiation between populations (F_{st}), a decrease in the effective breeding population size (N_e) between sampling sites on either side of Interstate 88, and limited gene flow between populations on either side of the highway are expected. Here, microsatellite data are used to examine these critical genetic parameters.

Materials and Methods

Study sites: Contemporary ($n = 26$) and historic ($n = 38$) data were collected from two streams in Otsego County and two streams in Delaware County, New York. The furthest sections of the two Otsego County streams sampled are rectilinearly 11 km and 28 km north of Interstate 88. The furthest sections of the two Delaware county streams sampled are rectilinearly 3 km and 9 km south of Interstate 88. All streams sampled are part of the Susquehanna watershed, and terminate on the southern side of Interstate 88 (Fig. 1). Contemporary sites were sampled during the spring/early summer and late fall active periods of 2015 and 2016 using the Regional Conservation Needs protocol, which involves sampling in 1 mi increments (Jones et al. 2015).

Samples: Blood samples were used for contemporary data, and 1 mm tail tips were harvested from dried historic specimens. Blood samples (0.1–0.5 ml) were collected from the dorsal coccygeal vein using a sterile 1.0 ml 25-gauge syringe (Jones et al. 2015). Blood was transferred into test tubes immediately upon return from the field and stored in 1:1 1 x PBS buffer in a -20 °C freezer. Genomic DNA was extracted from each sample using the QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, California, USA). Tail tips were digested in Proteinase K for 36 h. Each extracted sample was stored in a -20 °C freezer. Seven microsatellite loci were examined (GmuD16 [Genbank accession number: AF516235], GmuD40 [AF517244], GmuD51 [AF517239], GmuD87 [AF517244], GmuD88 [AF517245], GmuD93 [AF517248], and GmuD95 [AF517249]) using primers initially designed for a close relative of the Wood Turtle, *Glyptemys muhlenbergii* (King and Julian 2004). Samples were amplified using the QIAGEN Multiplex PCR kit (Qiagen, Inc., Valencia, California, USA) and a modified version of the PCR protocol (Castellano et al. 2009). The length of the extension step from this protocol was doubled to optimize historic sample amplification due to the highly-fragmented nature of this DNA. PCR products were analyzed at the Cornell Biotech Institute in Ithaca, New York, and visualized using GENEMARKER version 2.6.7 (Hulce et al. 2011).

Statistical analysis: MICROCHECKER version 2.2.3 was used to test each locus for the presence of null alleles, scoring errors, and large allele dropout (Van Oosterhout et al. 2004). Clustering was used to assign individuals to populations using the program STRUCTURE version 2.2 (Pritchard et al. 2000; Falush et al. 2003). Data were analyzed for all contemporary and historic turtles from each of the sites north (Otsego County) and south (Delaware County) of Interstate 88. For each analysis, three runs were used for each value of K (number of assumed populations) ranging between one and nine.

A 106 burn-in period was used, and 106 Markov Chain Monte Carlo (MCMC) iterations were used in the default “admixture model” of ancestry and correlated allele frequencies. Population origin data (north and south) were provided for each individual. Mean log likelihood and DK values were used to assign individuals to populations [K] (Evanno et al. 2005).

Deviations from the Hardy-Weinberg (HWE) expectation among pairs of loci were tested along with mean heterozygosity, allelic richness, numbers of private alleles, inbreeding coefficient (F_{is}), genetic differentiation (F_{st}), and effective population size (N_e) using GenAEx version 6.5 (Peakall and Smouse 2006, 2012) for the populations north (Otsego) and south (Delaware) of Interstate 88. The comparison of historic and contemporary loci F_{is} was made using a student's t -test. Evidence of a bottleneck on the contemporary data was tested using the program BOTTLENECK (version 1.2.02, Cornuet and Luikart 1996) with an infinite allele model (IAM) and the two-phase model (TPM) recommended by Luikart et al. (1998) over 10,000 iterations. The significance of Wilcoxon test score output ($\alpha = 0.05$) and mode shift were both used as evidence of a bottleneck (Cornuet and Luikart 1996; Luikart et al. 1998; Chiuicchi and Gibbs 2010). Short-term migration (m) between the “last few generations” was estimated using Bayesian inference software BAYESASS (version 3.03, Wilson and Rannala 2003) using 3×10^7 iterations with two long-chains sampling every 2,000 iterations, and this included a burn-in of 107. Specifically, a time span reaching back < 5 generations, or 25–125 years (Chiuicchi and Gibbs 2010) was used since the estimated generation time for Wood Turtles is 25 years (Farrell and Graham 1991; Galois and Bonin 1999). Multiple independent model runs were made using a random seed, with final selection made based on Maximum Likelihood (e.g., Chiuicchi and Gibbs 2010).

Results

Six of the seven microsatellites amplified consistently. The exception was GmuD51, which was removed from further analyses. Historic specimens had a high allele dropout rate (46%), which is expected for highly fragmented antique DNA (Mills et al. 2000; Sefc et al. 2003). No evidence of genotyping error or null alleles was found for those that amplified. Sample size, effective population size, heterozygosity, and overall differentiation between north and south populations, for both historic and contemporary data, are summarized in Table 1. F_{st} values for the contemporary populations was 0.166, while historically they were estimated at 0.081 (Table 2). Heterozygosity estimates per locus are summarized in Table 2. Northern contemporary and historic data each showed three of six loci out of HWE. Southern contemporary data displayed four of six loci

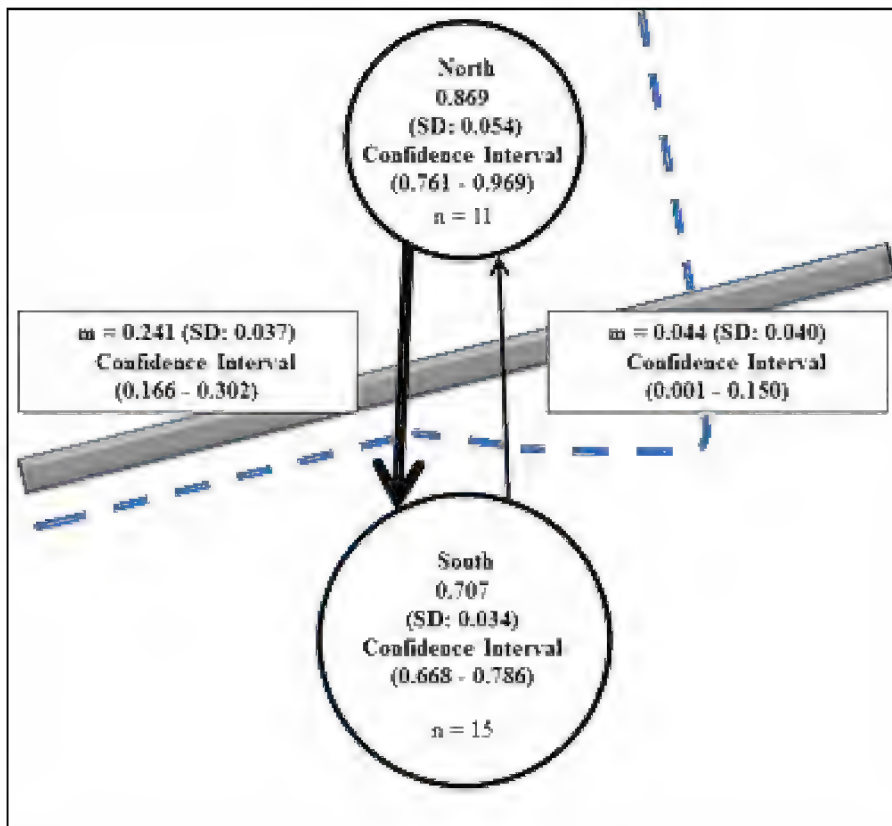


Fig. 2. Estimate of short-term gene flow among populations north and south of Interstate Highway 88 (gray bar) and the Susquehanna River (dashed line) shown with 95% confidence intervals. Circle size reflects relative sample size. Values inside of circles represent the contribution of gene flow from within populations.

out of HWE, while the southern historic data displayed a single locus out of HWE. Comparison of historic and contemporary inbreeding (F_{is}) indicated no difference between estimations ($P = 0.30$). Fixation indexes for each locus are summarized in Table 3.

Contemporary samples clustered into two populations ($K = 2$, $\text{Ln P(D)} = -659.5$, $\text{Var} [\text{LnP(D)}] = 74.5$), with a clear distinction between north and south samples. Historic samples consistently clustered into a single population ($K = 1$, $\text{LnP(D)} = -787.9$, $\text{Var} [\text{LnP(D)}] = 33.0$). There was a deficiency of heterozygosity ($P = 0.04$) for the northern contemporary population under the Wilcoxon rank sign test in the TPM model. Specifically, none of the northern loci were in mutation-drift equilibrium, as five of six loci showed signs of heterozygosity deficiency with the final locus in excess under the IAM model. Southern contemporary populations showed no sign of a genetic bottleneck. Short-term migration (m) was conservatively estimated

to be higher going from north to south (24%) than south to north (4.4%) [Fig. 2].

Discussion

Genetic changes and trends in turtle populations may be difficult to detect due to their naturally long generation times and long lives (Gibbs and Amato 2000). Using the genetic material available from historic samples allowed the successful detection of changes between the populations across a relatively brief period of time. Although this study used a limited number of microsatellites (six of seven), the polymorphic nature of the markers and highly differentiated level of the populations suggest that the results capture an adequate amount of information for comparative genetic analysis (Kalinowski 2002, 2005; Arthofer et al. 2018), especially given the sample sizes for the historic and contemporary populations (Hale et al. 2012). Specifically, the results indicated that these local populations have likely become genetically fragmented over the last 60 years. This may indicate that certain freshwater turtle populations are more vulnerable, in terms of the rate of change, to shifts in genetic structure than previously thought.

Structural analysis of the contemporary data revealed that Wood Turtle populations clustered into two populations, where historically, they were likely a single interconnected unit. In addition, the same samples revealed that local populations have become more differentiated over time as an increase of F_{st} was observed from 0.081 in historical to 0.166 in contemporary populations. This shift from moderate differentiation (> 0.05) to great differentiation (> 0.1) over an evolutionarily short period of time would appear to be aberrant when compared to previous studies examining Wood Turtle differentiation (Hartl and Clark 1997). However, Tessier et al. (2005) sampled Wood Turtles in a similar semi-montane habitat, and found that some of their populations, which had a proximity between sites comparable to those in the current study (~15–50 km), had similar differentiation as the historic samples studied here. Conversely, the contemporary sample differentiation found here is more similar to that of their sites which were much further apart

Table 1. Summarized outputs of population parameters from GenAlEx v 6.5. Parameters displayed are sample size (n), effective population size (N_e), observed (H_o) and expected (H_e) heterozygosity, and overall differentiation (F_{st}) between North and South collecting sites.

Historic (1955-1965)							
	n	N_e (SE)	Mean # alleles (SE)	H_o (SE)	H_e (SE)	Private alleles	F_{st}
North	20	6.9 (1.5)	10.0 (1.5)	0.61 (0.11)	0.82 (0.04)	40	0.081
South	18	5.3 (1.1)	7.8 (1.6)	0.65 (0.14)	0.77 (0.04)	27	
Total	38	12.2					
Contemporary (2015-2016)							
North	11	4.0 (0.4)	6.3 (0.5)	0.67 (0.04)	0.74 (0.02)	12	0.166
South	15	7.0 (0.8)	11.0 (0.8)	0.81 (0.04)	0.85 (0.02)	19	
Total	26						

Table 2. Summarized contemporary observed (H_o) and expected (H_e) heterozygosity.

Locus	Size range (bp)	# of alleles	North		South	
			H_o	H_e	H_o	H_e
GmuD16	165–296	24	0.700	0.830	0.857	0.829
GmuD87	238–394	24	0.700	0.840	0.857	0.768
GmuD88	114–264	30	0.733	0.849	0.867	0.838
GmuD93	125–389	20	0.333	0.736	0.714	0.885
GmuD95	122–266	24	0.778	0.833	0.667	0.871
GmuD40	157–280	21	0.778	0.747	0.917	0.892

(> 60 km) than the sites in this analysis (Tessier et al. 2005). Overall, this suggests the populations examined here, which are relatively close to one another in terms of physical distance, possess genetic differentiation that is more akin to areas further apart, implying that there is a barrier preventing genetic exchange between them. In flatter areas along the coastal plains of the northeastern United States, differentiation among Wood Turtle populations is essentially non-existent, enabling populations to be panmictic across separation distances greater than 40 km (Castellano et al. 2009). This leads us to believe that dispersal limitation is due to some environmental factor, and not simply life history.

A study on another terrestrial emydid turtle, *Terrapene ornata* (Ornate Box Turtle), in Texas found that a major highway built in 1937 was likely the cause of significant differentiation between populations on either side, but not the cause of a change in overall structure (Richtsmeier et al. 2008; Cureton et al. 2014). Similarly, Tessier et al. (2005) found that the St. Lawrence River acts as a barrier between Wood Turtles on either side of its shores, separating them structurally. It is possible that the observed structural separation between the populations studied here has been compounded by the combination of the intertwining bisection of Interstate 88, and the Susquehanna River (Fig. 1).

Despite the findings of Tessier et al. (2005), the migration analysis output in this study (Fig. 2) may suggest that flooding events are allowing at least some unidirectional gene flow to persist between the populations on either side of Interstate 88. In short, local hydrology from lower order streams at both the north

and south sampling sites terminate at the Susquehanna River south of Interstate 88. Research by Jones and Sievert (2009) indicates that flooding events, which have dramatic impacts on Wood Turtles, may play a vital role in connectivity. Specifically, flood events can displace a substantial (40%) portion of Wood Turtle subpopulations by long distances (1.4–16.8 km) downstream (Jones and Sievert 2009), which may explain the unidirectionality of the migration estimates found here (Fig. 2).

This possibility seems even more likely when considering the local footprint of Interstate 88, much of which is built on steep and sometimes craggy mounds protruding from the stream and forest surface. These mounds, which span four total lanes and occasionally split with a center depression or open fall at the median strip, most likely make terrestrial genetic exchange near impossible between the north and south populations. Moreover, the Susquehanna in its entirety is substantially narrower than the St. Lawrence River (Kammerer 2005), which may make survival of flooding events more likely. Additionally, we observed Wood Turtles using the Susquehanna's embankments and flood plains with some regularity, so although its flow may prevent and influence movement, it should not be considered an insurmountable genetic barrier like the much larger St. Lawrence (Tessier et al. 2005). As such, it is likely that Wood Turtle movement is influenced by the directionality of the flow in creeks and rivers, and may explain the unidirectional migration observed here (Fig. 2).

Although some streams and creeks in Delaware County run north to south as they percolate down from the Catskill mountains, the northern Delaware drainages

Table 3. Microsatellite fixation indexes for both historic and contemporary populations.

Locus	Historic			Contemporary		
	F_{is}	F_{it}	F_{st}	F_{is}	F_{it}	F_{st}
GmU16	0.245	0.308	0.084	0.093	0.226	0.146
GmU87	-0.246	-0.045	0.161	-0.056	0.102	0.150
GmU88	0.253	0.282	0.038	0.030	0.157	0.131
GmU93	0.956	0.961	0.110	0.117	0.367	0.283
GmU95	0.136	0.171	0.040	0.166	0.270	0.125
GmU40	-0.028	0.024	0.051	-0.048	0.117	0.158
Mean	0.220	0.284	0.081	0.050	0.206	0.166
SE	0.166	0.147	0.020	0.037	0.041	0.024

near the sampling sites used in this study flow south to north, terminating into the Susquehanna. Previous research by Brown et al. (2016) indicated that Wood Turtles become more terrestrial as the thermoregulatory benefits of returning to the water at night diminish during the summer, but they never seem to stray too far from flowing water. Furthermore, a species of turtle that divides its time between land and water (Kaufmann 1992) is expected to use smaller rivers as corridors like many other turtle species (Gibbs and Amato 2000). If local Wood Turtles are using the Susquehanna River as a corridor at least in part, with strong flow and flooding events acting as a migration regulator, further genetic research should yield an F_{st} gradient and not complete differentiation. In other words, central New York's populations should be progressively more differentiated from populations further south along the Susquehanna River, but not completely differentiated altogether. Therefore, further research should investigate the potential of large rivers, namely the Susquehanna River, to act as turtle barriers or corridors.

If the Susquehanna is acting as a unidirectional barrier, long-term declines could prove problematic for the local populations. Specifically, the loss of only a few individuals may appear to be minimal in terms of allelic diversity, but a negative change in the effective breeding population (N_e), as was observed, could prove to have adverse conservation consequences. For example, similar rates of reduction in Bog Turtle populations have been identified as substantially increasing the likelihood of extirpation (Shoemaker 2011). Certain life histories are also known to be susceptible to such impacts (Jonsson and Ebenman 2001). Specifically, for Bog Turtle populations, the loss of only a few breeding adult individuals can have greater impacts on populations than even dramatic short-term increases in juvenile mortality (Shoemaker 2011). Similar losses for Wood Turtles, the closest known relative of the Bog Turtle, could prove to be equally problematic. This vulnerability, again, would be due to their long generation times, high juvenile mortality rate, and reliance on adult survival to bolster the populations (Gibbs and Amato 2000). This situation presents a suite of unique conservation issues which are likely to also be applicable to other freshwater turtle species.

One noteworthy observation here is that the bottleneck analysis presented identified both heterozygosity deficiency and excess in the northern populations. Typically, heterozygosity deficiency is associated with a founder effect (Cornuet and Luikart 1996) or possibly the existence of a subpopulation structure within the sample, known as the Wahlund effect (Wahlund 1928). However, in rare situations when allelic diversity is high, as it is with the Wood Turtles in this study, heterozygosity deficiency can be the result of post-bottleneck changes, such as mutation or population expansion, which fill the allelic gaps left by limited random selection (Cornuet and

Luikart 1996; Maruyama and Fuerst 1985). For freshwater turtle populations, which typically have a small number of long-lived adults possessing the majority of effective alleles (Crouse and Frazer 1995; Gibbs and Amato 2000), the loss and sequential replacement of these few valuable reproductive individuals appear to enable this particular scenario. Considering that none of the loci for the northern population were in mutation-drift equilibrium, and they showed evidence of deficiency and excess, it seems clear that something has impacted the northern population allelic ratios. Further research into Otsego County's populations north of Interstate 88 is required to determine the source of this irregularity. Additionally, we recommend that future management plans for Wood Turtle populations in central New York and other regions with montane-riverine mosaics consider the potential genetic complications associated with anthropogenic habitat fragmentation. To mitigate these potential impacts, the installation of appropriately sized culverts, drift nets, and turtle-crossing signs (Aresco 2005; Woltz et al. 2008), in high-density areas (Gunson and Schueler 2012), is necessary.

Conclusions

Consistent with other research (Steen and Gibbs 2004; Gibbs and Steen 2005; Steen et al. 2006) the observed division and reduction in N_e in the local populations studied here, the potential recent genetic bottleneck, the increased differentiation, and the overall change in population structure are most likely attributable to the additional fragmentation of the local montane/riverine habitat by the bisecting interstate highway. A clear north to south directionality of gene flow was observed from the short-term (25–125 years) migration estimate. The full implications of this dichotomy, in the context of potential isolation due to fragmentation, have yet to be determined. As a species of conservation concern, understanding the genetic landscape at the local and regional levels is vital for planning future management. In terms of conservation, it is possible New York's central populations hold unique alleles as they are surrounded by three major highways and two large mountain ranges. In turn, this may require that management efforts treat these isolated populations as demographically independent units, should they yield unique genetic variation. As such, we recommend that policy and management reflect the impacts that bisecting highways can have on populations within a local region, and not just those adjacent to a thoroughfare. Additionally, we recommend that policy and management efforts reflect the evidence, which suggests that hydrology may dictate Wood Turtle gene flow. Furthermore, research focused on determining where central New York's populations fit within the context of the entire region's genetic landscape will be particularly useful.

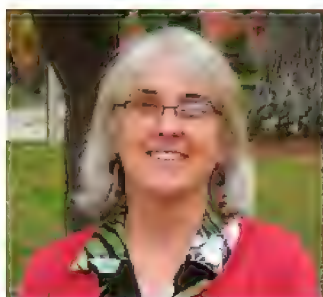
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Alex Robillard received a B.S. in Conservation Biology from State University of New York, College of Environmental Science and Forestry (SUNY-ESF, Syracuse, New York, USA), and an M.S. in Biology from SUNY-Oneonta. Currently a predoctoral fellow at the Smithsonian Data Science Lab and National Zoo, and a Ph.D. student in the Marine-Estuarine and Environmental Science Program at the University of Maryland, Alex's dissertation focus is on the conservation and population genetics of the Eastern Pacific Hawksbill Sea Turtle. His past research had focused on the ecology of the Bog Turtle, Wood Turtle, and Eastern Massasauga Rattlesnake in New York State. Alex is also researching how deep machine learning and computer vision can be used to combat the poaching of sea turtles.

Sean Robinson completed a B.A. at Hartwick College (Oneonta, New York, USA), an M.S. at SUNY-ESF, and a Ph.D. at the University at Albany, New York. Dr. Robinson joined the SUNY-Oneonta faculty in 2010 where his research is focused on understanding how the mode of reproduction in plants, particularly bryophytes, affects colonization of new habitats, range expansions, and the exchange of alleles both within and between populations. Additionally, Dr. Robinson conducts research focused on vegetation dynamics on alpine summits, using molecular techniques to identify population structuring.

Elizabeth Bastiaans completed a B.A. at the University of Chicago, a Ph.D. at the University of California, Santa Cruz, and a postdoctoral fellowship at the University of Minnesota, Twin Cities. Elizabeth joined the SUNY-Oneonta faculty in 2015. Her previous research focused on sexual signal evolution in Mexican montane lizards and life history evolution in tropical crickets. At SUNY-Oneonta, Dr. Bastiaans has started to focus her research on the reproduction and physiology of the Wood Turtle across New York State, while maintaining her previous collaborations with colleagues in Mexico.

Donna Vogler was born and educated in the Midwestern United States, with a B.S. from The Ohio State University, and an M.S. from Iowa State University, before working for the U.S. Fish and Wildlife Service in Washington, DC. Donna earned a Ph.D. from Penn State University in Botany, and was a post-doctoral researcher at the University of Pittsburgh before joining the SUNY-Oneonta faculty in 2000. Dr. Vogler's recent research topics include demographic studies of invasive plant species (e.g., Marsh Thistle, *Cirsium palustre*), floral mechanisms related to self vs. outcross pollination, and using Wood Turtle habitat communities and vegetation management at regional airports to reduce wildlife hazards.